NOTE *Virology*

Genetic Analysis of an H5N2 Highly Pathogenic Avian Influenza Virus Isolated from a Chicken in a Live Bird Market in Northern Vietnam in 2012

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ABSTRACT. In August 2012, A/chicken/Vietnam/OIE-2215/2012 (H5N2) was isolated from a chicken in a live bird market (LBM) in Northern Vietnam. Intravenous pathogenicity test revealed that this virus is highly pathogenic in chickens. The PA, HA, NP and M, PB2 and NA, and PB1 and NS genes of the isolate were phylogenetically closely related to those of A/duck/Vietnam/OIE-2202/2012 (H5N1) of clade 2.3.2.1, A/chicken/Vietnam/OIE-1611/2012 (H9N2) and A/chicken/Vietnam/OIE-2468/2012 (H9N2), respectively. All of these viruses were isolated from birds in LBMs in the same province. These results indicate that A/chicken/Vietnam/OIE-2215/2012 (H5N2) is a genetic reassortant and that surveillance of avian influenza in LBMs and stamping out policy are essential for the eradication of highly pathogenic avian influenza viruses from Asia.

KEY WORDS: avian influenza, H5N2, live bird market, reassortant, Vietnam.

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In 1996, the first outbreak of H5N1 highly pathogenic avian influenza virus (HPAIV) infection occurred at a goose farm in Guangdong Province, China [12]. Since then, H5N1 HPAIV has spread and circulated in poultry in Eurasia and Africa [9]. In Northern Vietnam, H5N1 viruses of clade 2.3.2.1 have been endemic in live bird markets (LBMs) since 2011 [10]. H9N2 avian influenza viruses have also caused outbreaks in poultry, resulting in serious economic losses in Asia and the Middle East since the 1990s [2, 4, 14]. The co-circulation of multiple influenza viruses lead to genetic reassortment between them, expanding the genetic diversity of these viruses. Internal genes of A/Hong Kong/156/1997 (H5N1) are closely related to A/teal/Hong Kong/W312/1997 (H6N1) and A/quail/Hong Kong/G1/1997 (H9N2) [1, 5]. A/chicken/Hebei/1102/2010 (H5N2), A/duck/Eastern China/1111/2011 (H5N2) and A/duck/Eastern China/008/2008 (H5N5), which were isolated from poultry, are reassortants of H5N1 viruses of clade 7 or clade 2.3.4 with other subtypes, such as H9N2, H3N2 or H6N5 influenza viruses [3, 13].

In the present study, surveillance of avian influenza was performed in households, LBMs, slaughterhouses and bird sanctuaries in Northern and Southern Vietnam from 2009 to 2012. A total of 5,421 tracheal and cloacal swab samples were collected from poultry, and 266 influenza viruses were isolated. In our previous study, the subtypes of these isolates were identified as H3N2, H3N6, H3N8, H4N2, H4N6, H5N1, H5N2, H6N2, H6N6, H6N9, H7N1, H9N2, H9N6, H9N8, H10N7, H11N3 and H11N9, and genetic and antigenic analyses were performed on the H5, H3, H6 and H9 influenza viruses [7, 10]. In the present study, an H5N2 virus isolated from an apparently healthy chicken in Northern Vietnam in August 2012 was phylogenetically analyzed.

Tracheal and cloacal swab samples collected from a chicken in a LBM in North Vietnam 2012 were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. After incubation at 35°C for 30–48 hr, allantoic fluid showing hemagglutination activity was collected and stored at −80°C. Subtype identification of the isolate was done by hemagglutination-inhibition and neuraminidase-inhibition tests [6]. Viral RNA of A/chicken/Vietnam/OIE-2215/2012 (H5N2) was extracted from the allantoic fluids of embryonated chicken eggs, and direct sequencing of the viral genes was performed. The accession numbers of the sequences registered at GenBank/EMBL/DDBJ are shown in Table 1. Sequence analysis of the hemagglutinin (HA) cleavage site revealed that the deduced amino acid sequence of the C-terminus of HA1 had multiple basic amino acid residues (RERRRKR/GLF), which is characteristic of HPAIV according to the manual of the World Organization for Animal Health [8]. To assess its intravenous pathogenicity in chickens, 0.2 m*l* of a 1/10 dilution of the fresh allantoic fluid of chicken embryo infected with the isolate (256 HA

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Table 1. Genetic identities to other influenza A viruses of A/chicken/Vietnam/OIE-2215/2012 (H5N2)

Gene segment	Accession No.	Virus with the highest identity	Identities (%)
PB2	AB766236	A/chicken/Vietnam/OIE-1611/2012 (H9N2)	100
PB1	AB766237	A/chicken/Vietnam/OIE-2468/2012 (H9N2)	95.9
PA	AB766238	A/duck/Vietnam/OIE-2202/2012 (H5N1)	100
HA	AB766239	A/duck/Vietnam/OIE-2202/2012 (H5N1)	99.9
NP	AB766240	A/duck/Vietnam/OIE-2202/2012 (H5N1)	99.9
NA	AB766241	A/chicken/Vietnam/OIE-1611/2012 (H9N2)	99.8
M	AB766242	A/duck/Vietnam/OIE-2202/2012 (H5N1)	100
NS	AB766243	A/chicken/Vietnam/OIE-2468/2012 (H9N2)	98.9

Only the influenza viruses with the highest degree of nucleotide sequence identity based on the GenBank/EMBL/DDBJ nucleotide BLAST search analysis are included in the table.

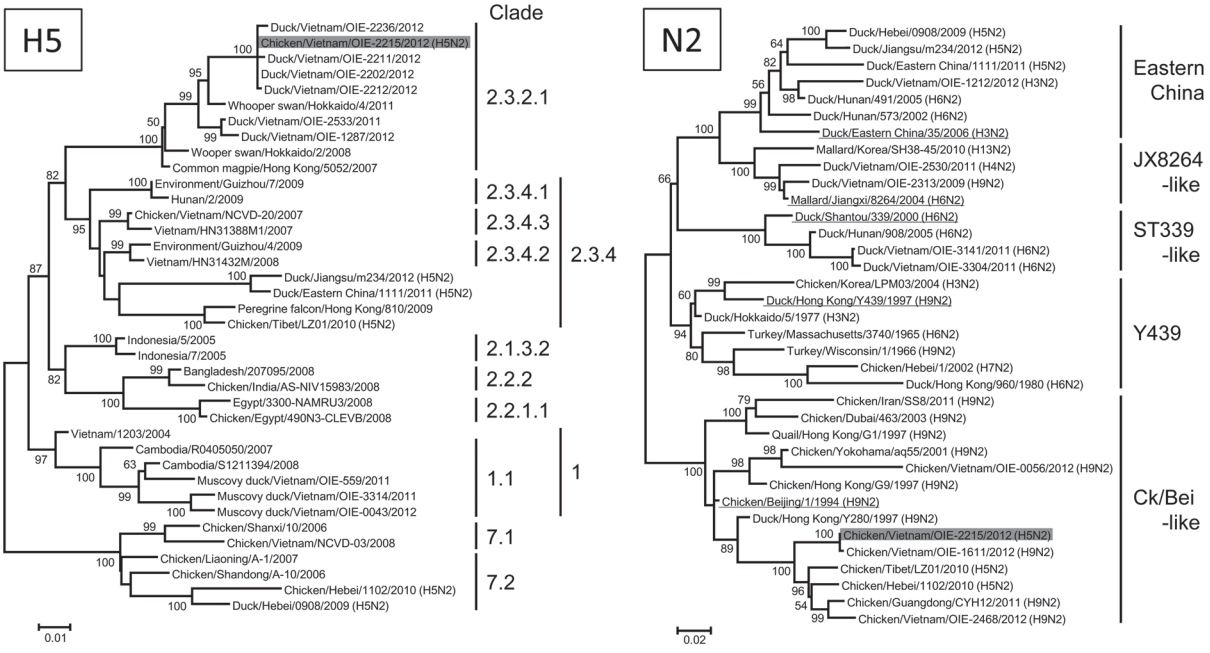

Fig. 1. Phylogenetic trees for the H5 HA and N2 NA genes of influenza viruses Nucleotides 49–1,019 (971bp) of the HA gene and 195–1,363 (1,169 bp) of the NA gene were analyzed by the maximum-likelihood method using MEGA 5.0 software (http://www.megasoftware.net/). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The virus isolated in this study is highlighted in gray. H5 HA sequences are classified into genetic clades defined by the WHO/OIE/FAO H5N1 Evolution Working Group [11]. HA and NA subtypes were eliminated from the names of H5N1 viruses. Representative viruses in each N2 NA lineage are underlined.

units) was inoculated intravenously into eight 7-week-old chickens (*Gallus gallus*) according to the standard protocol [8]. Each bird was housed in self-contained isolator units (Tokiwa kagaku, Tokyo, Japan) at an animal biosafety level 3 laboratory facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Hokkaido University (approval number: 11–0087), and all experiments were performed according to the guidelines of this committee. All chickens died within 24 hr, and the isolate was defined as HPAIV.

From the phylogenetic analysis, the HA gene of the H5N2 virus was classified into the genetic clade 2.3.2.1 (Fig. 1), according to nomenclature defined by the WHO/OIE/FAO H5N1 Evolution Working Group [11]. And, the PA, NP and M genes were closely related to those of H5N1 viruses, which are endemic in East Asia and Southeast Asia and were almost identical to those of A/duck/Vietnam/OIE-2202/2012 (H5N1) (Table 1). In contrast, the NA gene was classified into the A/chicken/Beijing/1/1994 (H9N2)-like lineage (Fig. 1). And, the PB2, PB1 and NS genes were closely related to those of the H9N2 viruses isolated from chickens in China

and were highly homologous to A/chicken/Vietnam/OIE-1611/2012 (H9N2) and A/chicken/Vietnam/OIE-2468/2012 (H9N2) (Table 1). The H5N1 and H9N2 viruses which are genetically related to the present H5N2 virus were isolated from birds in LBMs in the same province. These results indicate that the chicken was co-infected with the H5N1 and H9N2 viruses and generated the genetic reassortant, A/chicken/Vietnam/OIE-2215/2012 (H5N2).

Recently, H5N2 or H5N5 HPAIVs which were generated by reassortment between H5N1 viruses and influenza viruses of other subtypes were isolated from poultry in Asia [3, 13]. In Asia, LBM is an ideal environment for genetic reassortment events and interspecies transmission of avian influenza viruses. It is, therefore, important to expand surveillance and improve the hygienic control measures of LBM to eradicate highly pathogenic avian influenza in Asia.

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REFERENCES

1. Chin, P. S., Hoffmann, E., Webby, R., Webster, R. G., Guan, Y., Peiris, M. and Shortridge, K. F. 2002. Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. *J. Virol.* **76**: 507–516. [Medline] [CrossRef]
2. Golender, N., Panshin, A., Banet-Noach, C., Nagar, S., Pokamunski, S., Pirak, M., Tendler, Y., Davidson, I., García, M. and Perk, S. 2008. Genetic characterization of avian influenza viruses isolated in Israel during 2000–2006. *Virus Genes* **37**: 289–297. [Medline] [CrossRef]
3. Gu, M., Liu, W., Cao, Y., Peng, D., Wang, X., Wan, H., Zhao, G., Xu, Q., Zhang, W., Song, Q., Li, Y. and Liu, X. 2011. Novel reassortant highly pathogenic avian influenza (H5N5) viruses in domestic ducks, China. *Emerg. Infect. Dis.* **17**: 1060–1063. [Medline]
4. Guan, Y., Shortridge, K. F., Krauss, S. and Webster, R. G. 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc. Natl. Acad. Sci. U. S. A.* **96**: 9363–9367. [Medline] [CrossRef]
5. Guan, Y., Shortridge, K. F., Krauss, S., Chin, P. S., Dyrting, K. C., Ellis, T. M., Webster, R. G. and Peiris, M. 2000. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *J. Virol.* **74**: 9372–9380. [Medline] [CrossRef]
6. Kida, H. and Yanagawa, R. 1979. Isolation and characterization of influenza A viruses from wild free-flying ducks in Hokkaido, Japan. *Zentralbl. Bakteriol. Orig. A* **244**: 135–143. [Medline]
7. Nomura, N., Sakoda, Y., Endo, M., Yoshida, H., Yamamoto, N., Okamatsu, M., Sakurai, K., Hoang, N. V., Nguyen, L. V., Chu, H. D., Tien, T. N. and Kida, H. 2012. Characterization of avian influenza viruses isolated from domestic ducks in Vietnam in 2009 and 2010. *Arch. Virol.* **157**: 247–257. [Medline] [CrossRef]
8. OIE—World Organization for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 "Avian Influenza". http://www.oie.int/eng/normes/mmanual/A_summry.htm.
9. OIE—World Organization for Animal Health Update on Avian Influenza, http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/.
10. Okamatsu, M., Nishi, T., Nomura, N., Yamamoto, N., Sakoda, Y., Sakurai, K., Chu, H. D., Thanh, L. P., Nguyen, L. V., Hoang, N. V., Tien, T. N., Yoshida, R., Takada, A. and Kida, H. 2013. The genetic and antigenic diversity of avian influenza viruses isolated from domestic ducks, muscovy ducks, and chickens in northern and southern Vietnam, 2010–2012. *Virus Genes* **47**: 317–329. [Medline] [CrossRef]
11. WHO/OIE/FAO H5N1 Evolution Working Group. 2012 Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. *Influenza Other Respi. Viruses* **6**: 1–5. [Medline] [CrossRef]
12. Xu, X., Subbarao, Cox, N. J. and Guo, Y. 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* **261**: 15–19. [Medline] [CrossRef]
13. Zhao, G., Gu, X., Lu, X., Pan, J., Duan, Z., Zhao, K., Gu, M., Liu, Q., He, L., Chen, J., Ge, S., Wang, Y., Chen, S., Wang, X., Peng, D., Wan, H. and Liu, X. 2012. Novel Reassortant highly pathogenic H5N2 avian influenza viruses in poultry in China. *PLoS One* **7**: e46183. [Medline] [CrossRef]
14. Zhou, J. P., Ge, F. F., Liu, J., Ju, H. B., Yang, D. Q., Wang, J., Zhang, W. Y. and Liu, P. H. 2012. Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai, China, from 2006 to 2010. *Arch. Virol.* **157**: 1193–1198. [Medline] [CrossRef]